Available online at www.sciencedirect.com

ScienceDirect

Procedia Chemistry 2 (2010) 34–45

Procedia Chemistry

www.elsevier.com/locate/procedia

5th Conference by Nordic Separation Science Society (NoSSS2009)

Stability study of somatropin by capillary zone electrophoresis

Thore W. Wilhelmsen[a], Venke Skibeli[b], Finn Cato Arntzen[a]

Norwegian Medicines Agency, Sven Oftedals vei 6, N-0950 Oslo, Norway
[a]Biological Laboratory, [b]Section for Safety and Clinical Evaluation

Abstract

Human Growth Hormone (hGH) is a monomeric 22 kilo Dalton (kDa), 191 amino acid protein with an isoelectric point (pI) close to pH 5, produced in the anterior pituitary gland. High level production of somatropin (recombinant hGH) is done in Escherichia coli (E. coli) to meet the demand and to avoid possible Creutzfeldt-Jacob disease (CJD).
The present study was initiated on the basis of results from post-marketing control of all somatropin preparations on the Norwegian market. The samples consisted of preparations presented as somatropin in solution, and some freeze-dried preparations were also included for comparison.
The present study showed a significant degree of degradation of somatropin in solution. Deamidation increased over time for preparations in solution, as well as for freeze-dried preparations after dissolution. Preparations in solution showed high content of deamidated and cleaved forms. Freeze-dried preparations after dissolution and storage showed high content of deamidated forms, but low content of cleaved forms. Also, in one preparation, an unknown peak was detected in the electropherogram from capillary zone electrophoresis (CZE), eluting after the principal peak, in front of the Gln-18 somatropin peak.

Keywords: Somatropin; degradation; deamidation; biological effect; glycosylation; capillary zone electrophoresis.

1. Introduction

Mammalian growth hormones (GH) are heterogeneous proteins consisting of several isoforms and variants. The sources of the variety in forms reside at the level of the genome, mRNA splicing, post-translational modification and metabolism [1]. The 22 kDa form of human GH (hGH), first isolated in 1956 [2] and characterized in 1971 [3], is the main and most abundant form of GH in the pituitary gland. Post-translational modifications of 22 kDa GH include an amino-acylated form and two deamidated forms [4]. The deamidation occurs at amino acid positions 137 and 152.

There are minor differences in somatogenic and metabolic bioactivity among the GH variants, depending on species and assay systems used. The deamidated and acylated forms have similar growth-promoting and diabetogenic activity in rodents [1], and also in an *in vitro* cell proliferation assay. However, there is limited

Corresponding author: T.W. Wilhelmsen. Tel.: +47 22 89 77 00 ; fax: +47 22 89 77 99.
E-mail address: tore.wilhelmsen@noma.no.

1876-6196
doi:10.1016/j.proche.2009.12.008

information available concerning the distribution of the biological activities of GH among the different variants. This is mainly due to the unavailability of these variants in pure form.

The different recombinant somatropin (rhGH) preparations on the market are mostly produced in E. coli, however, some of the products are also made in mammalian cell lines. This may confer structural modifications to somatropin preparations other than the ones resulting from production in bacteria.

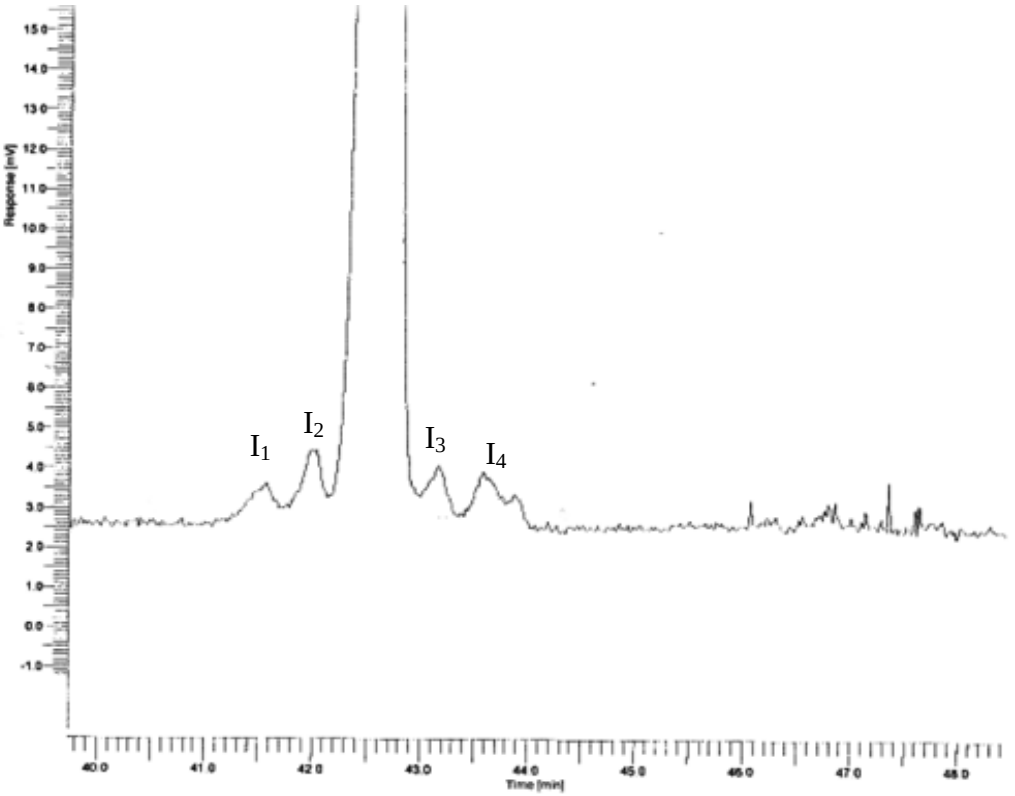
The prototype of what we today know as a capillary electrophoresis system (CE) was made in 1967 [5]. Electrophoresis in this system was based on a 1-3 mm rotating tube. The rotation was necessary to prevent sedimentation. In 1988 Browniee and Bio-Rad Laboratories introduced commercially available capillary electrophoresis equipment for capillaries <100 µm internal diameter (ID). Five years later there were ten producers of CE equipment world wide.

The following references have demonstrated a great application potential of capillary zone electrophoresis (CZE) for analysis of proteins and polypeptides in general, and for rhGH in particular [6-10].

CE was established as an analytical method in the European Pharmacopoeia (Ph. Eur.) in 2001 [11], and introduced in the monograph erythropoietin (EPO) concentrated solution in 2002 [12]. In 2006, the test for isoform distribution by isoelectric focusing (IEF) in the somatropin monographs was replaced by CZE [13].

Ph. Eur. describes the reference electropherogram of somatropin chemical reference substance (CRS) batch 2 made in E. coli (fig. 1) with two impurity peaks (I_1 and I_2), eluting prior to the principal peak, and at least two peaks (I_3 and I_4) eluting after the principal peak. I_2 corresponds to a cleaved form of somatropin, I_3 corresponds to Gln-18 somatropin and peak I_4 corresponds to the deamidated forms, eluting as a doublet. The British Pharmacopoeia (BP) [14] describes three peaks eluting after the principal peak; I_3, I_4 and I_5. Ph. Eur. and BP have defined the following

Fig. 1 Reference electropherogram of charged variants distribution of somatropin CRS batch 2 (Ph. Eur.).



I = impurity.
I_1, I_2 (cleaved form), I_3 (Gln-18 somatropin), I_4 (deamidated forms).

limits for these components: Maximum 6.5 % deamidated forms; for any other impurity, maximum 2.0 % is allowed of each, and maximum 11.5 % of total impurities (I_{Total}).

The present study was initiated on the basis of results from post-marketing control at the Norwegian Medicines Agency (NoMA) of all somatropin preparations available on the Norwegian market, sampled by the end of 2007. The samples consisted of preparations presented as somatropin in solution as well as freeze-dried preparations intended for reconstitution immediately before use, the latter corresponding to the Ph. Eur. monograph somatropin for injection [13].

The analytical results complied with the specifications of the Ph. Eur., except for the content of deamidated forms. At about one year before expiry date, the batches with somatropin in solution had an estimated content of desamido somatropin just above the limit of 6.5 % of the Ph. Eur. However, these results could not be considered as out of specification (OOS) results, because the Ph. Eur. monograph is not representative for the concerned pharmaceutical formulations, and the manufacturers' product specifications did not include the test for charged variants by CZE, nor describes any limit for the content of deamidated forms in their somatropin products.

Obviously, there is a need for a pharmacopoeia monograph on injectable somatropin marketed as a solution. The limits for the content of degradation products and the shelf life of the preparation will probably be a matter of consideration for the Ph. Eur. Commission in their work with revision of the somatropin monograph.

In the present study, to investigate the suitability of CZE to study degradation during storage, two brands of somatropin injection in solution available on the Norwegian market were tested. For comparative investigations also samples of the freeze-dried preparations of somatropin were tested at various time points after dissolution.

2. Experimental

2.1. Chemicals

Somatropin CRS batch 2 was obtained from the European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg, France. The somatropin reference was kept at -20 ^{o}C until use.

The somatropin preparations were obtained from a medicinal products wholesaler, Norsk Medisinaldepot, Oslo, Norway. Norditropin SimpleXx batch TR40418 was obtained from Novo Nordisk AS, Bagsværd, Denmark. The preparations were kept at 4 ^{o}C.

$(NH_4)_2HPO_4$, NaOH and ortho-Phosphoric acid 85 % were obtained from Merck, Darmstadt, Germany. Fluorinated liquid (FC-77) was obtained from 3M, Chemical Group, Haven, Belgium. Buffers and solutions were degassed before use at 3-6 bar and filtered through a 0.45 µm filter (Millex-HA, Millipore, Bedford, Massachusetts, USA). Milli-Q-H_2O (MQ-H_2O) was prepared with Elix 10, Milli-Q Synthesis A10 from Millipore Corporation, Bedford, Massachusetts, USA.

2.2. Handling of samples

Somatropin CRS batch 2, 1.69 mg of somatropin monomer, was dissolved in MQ-H_2O to a final concentration of 1.0 mg/ml.

Test samples in solution, Norditropin SimpleXx and NutropinAq, were diluted with MQ-H_2O to a final concentration of 1.0 mg/ml.

Freeze-dried samples were dissolved in their respective solvents and diluted with MQ-H_2O to a final concentration of 1.0 mg/ml.

All test solutions were kept at 4 ^{o}C.

2.3. Capillary zone electrophoresis (CZE) of somatropin samples

The BioFocus 3000 Capillary Electrophoresis System with Spectra Software version 3.00, Integration Software version 3.01, and BioFocus Capillary Cartridge from Bio-Rad Laboratories, California, USA, were used for the somatropin analysis. Uncoated fused silica capillaries from Polymicro Technologies, Arizona, USA, 74.8 cm x 50

µm ID (70.1 cm to the detection window) were used for the separation. The CZE experiments were performed in ammonium phosphate buffer 150 mM (19.81 g of $(NH_4)_2HPO_4$ was dissolved in 950 ml MQ-H_2O and titrated to pH 6.0 with ortho-Phosphoric acid 85 % and MQ-H_2O was added to 1000.0 ml). The individual experiments were performed at constant voltage (14 kV), giving a maximum current of 110 µA in the capillary.

All samples were applied hydrodynamically by pressure (2 p.s.i. x 1 s; 1 p.s.i. = 6894.76 Pa) and analysed with polarity from the positive to the negative electrode. The cartridge and the carousel were thermostated at 30 ^oC and 5 ^oC, respectively, by the Peltier thermoelectric cooling system with fluorinated liquid.

The washing procedure for the system between each run was as follows; initially, 100 mM sodium hydroxide for 120 s, then MQ-H_2O for 60 s, then electrophoresis buffer for 300 s, and finally MQ-H_2O for 0 s. Absorbance was monitored at 200 nm.

3. Results and discussion

Tables 1 and 2 give an overview of the somatropin preparations on the Norwegian market. Four preparations are freeze-dried formulations and two are presented as somatropin in solution. All, except one, of the preparations are prepared by recombinant DNA technology using E. coli as the host organism. For one product (Saizen) transformed mammalian cells are utilized. Tables 2.A and B show the composition of each preparation with declaration of ingredients and solvents. Detergents are included in the composition of the somatropin preparations in solution, probably to stabilize the product.

The distribution of charged variants in the somatropin preparations, as well as in somatropin CRS batch 2 were analysed at various time points after storage at 4 ^oC.

Fig. 2.A and B show the distribution profiles for somatropin CRS batch 2 solution immediately after preparation and after 285 days, respectively. Particularly, the content of impurity I_4, corresponding to the deamidated forms, showed a prominent increase during storage at 4 ºC. This is also demonstrated in fig. 3 (the x-axis is not linear). Tables 3.A and B show the content of charged forms for all preparations before and after storage of the test solutions. For all batches, except one, the level of deamidated forms (I_4) had raised above or were close to, the limit of 6.5 % defined in the somatropin for injection monograph, indicating a significant degradation. However, this limit is not formally applicable to the preparations of somatropin in solution (Norditropin SimpleXx and NutropinAq), and regarding the freeze-dried somatropin preparations these are not intended for storage after reconstitution.

Table 1. Somatropin preparations tested.

Product	Package	Expiry	Formulation	Production method
Norditropin SimpleXx:	10 mg/1.5 ml	09/2008	solution	rDNA, E.coli
	5 mg/1.5 ml	11/2008	solution	rDNA, E.coli
	5 mg/1.5 ml	12/2008	solution	rDNA, E.coli
	5 mg/1.5 ml	05/2009	solution	rDNA, E.coli
NutropinAq:	10 mg/2 ml	04/2009	solution	rDNA, E.coli
Genotropin:	0.4 mg	09/2008	freeze-dried	rDNA, E.coli
	5 mg	09/2009	freeze-dried	rDNA, E.coli
Humatrope:	12 mg	01/2009	freeze-dried	rDNA, E.coli
Saizen:	1.33 mg	05/2008	freeze-dried	rDNA, transformed mammalian cells
	8 mg	10/2009	freeze-dried	
Zomacton:	4 mg	11/2008	freeze-dried	rDNA, E.coli

Table 2.A Declared content of somatropin preparations on the Norwegian market: Solutions for injection.

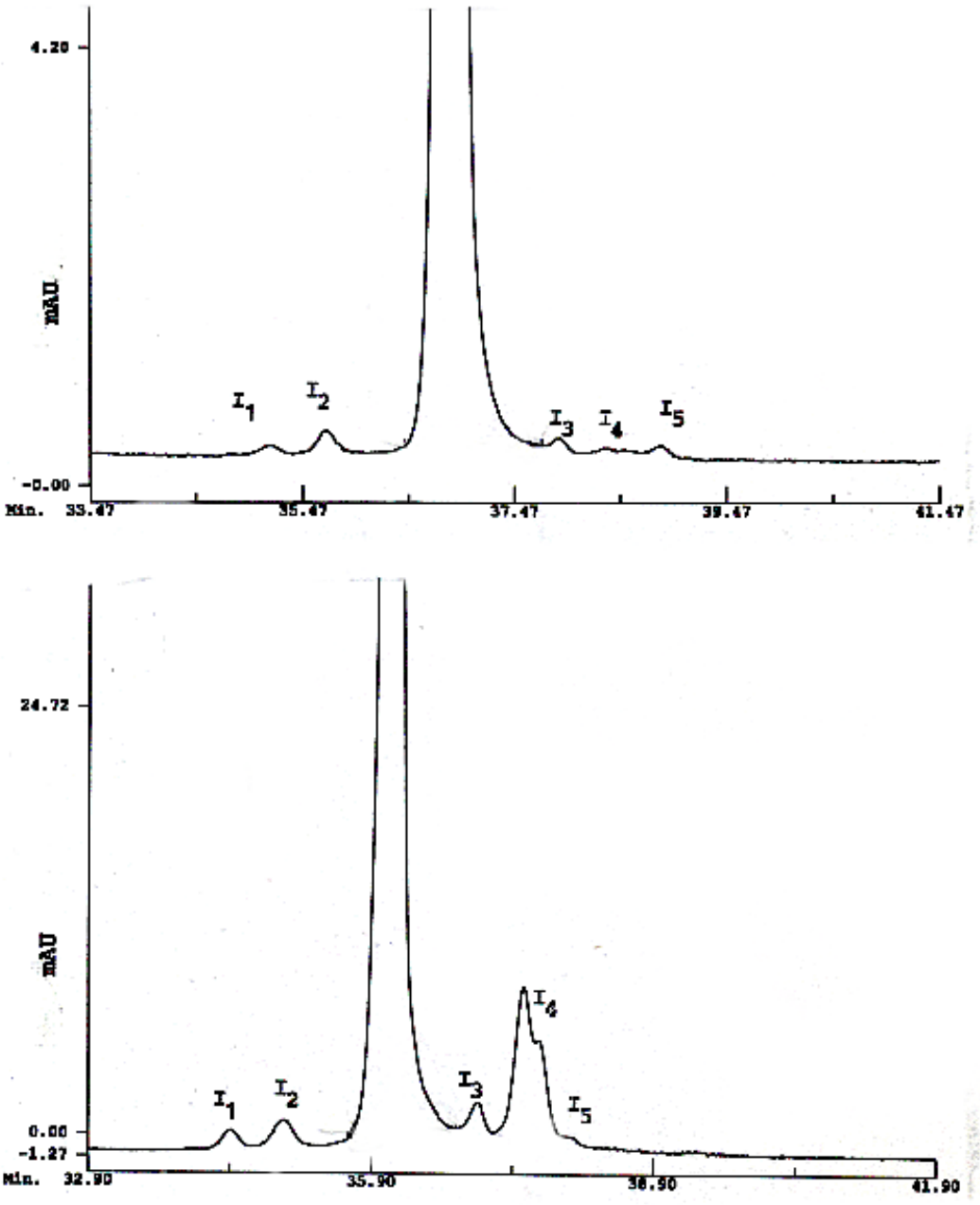
Product	rhGH mg	NaCl mg	Phenol mg	Polysorbate 20	NaCitrate mg	Citric acid mg	Histidine mg	Poloxamer 188	Mannitol mg	Aq. ml
Norditropin SimpleXx…:	5-15	-	x	-	-	-	x	x	x	1.5
NutropinAq:	10	x	x	x	x	x	-	-	-	2

Table 2.B Declared content of somatropin preparations on the Norwegian market: Freeze-dried preparations.

	Freeze-dried substance								Solvent				
Product	rhGH mg	Glycine mg	Mannitol mg	Saccharose mg	NaH_2PO_4 mg	Na_2HPO_4 mg	NaCl mg	NaOH mg	NaCl mg	Mannitol mg	Benzyl-alcohol mg	m-Cresol mg	Glycerol mg	Aq. ml
Genotropin:	0.2-2	0.21	1.04	-	0.045	0.025	-	-	-	11.5	-	-	-	0.25
	5	2	1.8	-	0.28	0.27	-	-	-	39	-	3	-	1
	12	2	12.2	-	0.41	0.4	-	-	-	27.8	-	3	-	1
Humatrope:	6	6	18	-	-	1.36*	-	-	-	-	-	9.45	53.55	3.15
	12	12	36	-	-	2.72*	-	-	-	-	-	9.45	9.14	3.15
	24	24	72	-	-	5.43*	-	-	-	-	-	9.45	9.14	3.15
Saizen:	1.33	-	20	-	x**	x***	x	-	9/ml	-	-	-	-	x
	3.33	-	5	-	x**	x***	-	-	9/ml	-	9/ml	-	-	5
	8	-	-	54.7	x**	x***	-	x	-	-	-	3/ml	-	1.37
Zomacton:	4	-	25.9	-	-	-	-	-	31.5	-	31.5	-	-	3.5

* = Na_2HPO_4 $7H_2O$, ** = NaH_2PO_4 H_2O, *** = Na_2HPO_4 $2H_2O$.
x = Amount/volume not given.
Poloxamer = Poloxamer 188 = Exocorpol, Pluronic F68.
Polysorbat 20 = Tween 20

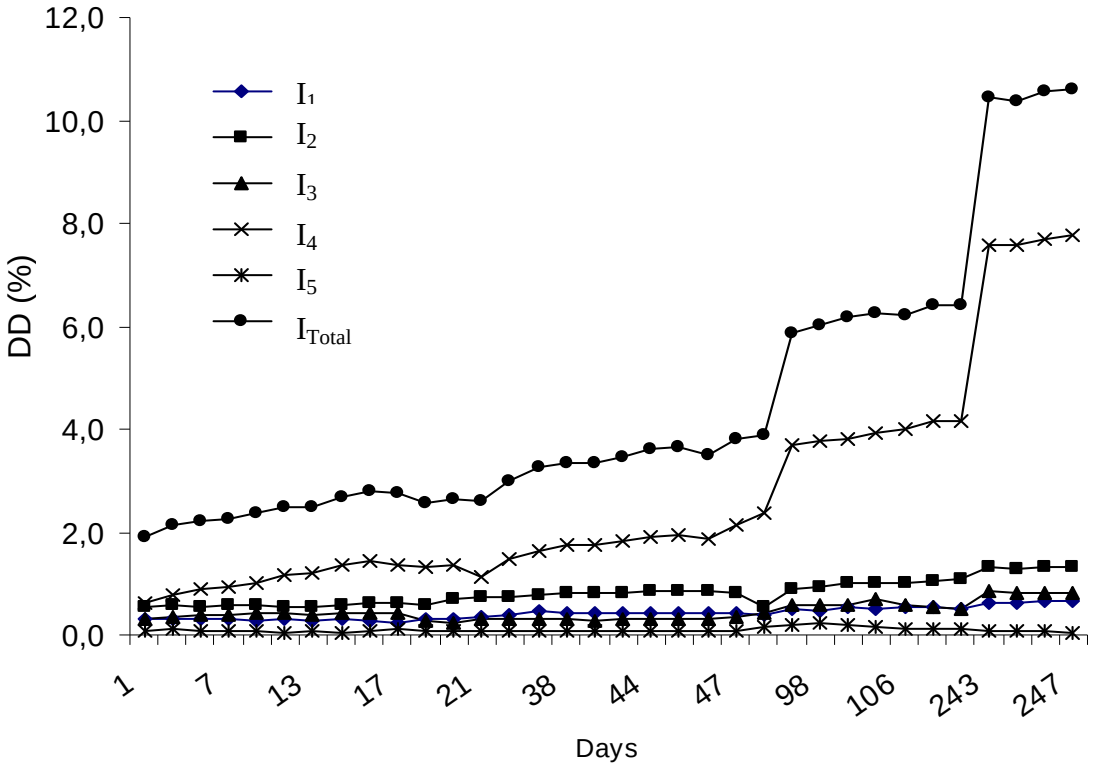
Fig. 2 Electropherogram of somatropin CRS batch 2, 1.0 mg/ml, freshly prepared (A) and after 285 days (B).



I = impurity.

I_1, I_2 (cleave B Gln-18 somatropin), I_4 (deamidated forms), I_5(deamidated form).

Fig. 3 Degradation of somatropin CRS batch 2, 1.0 mg/ml; monitored during storage at 4 °C.



I = impurity.
DD (%) = degradation degree in per cent.

The degradation of each preparation of somatropin in solution is shown in fig. 4.A-E. A steady increase in the level of degraded forms of somatropin (desamido forms) is demonstrated over time for all products. For comparison, degradation of each of the reconstituted freeze-dried preparations is shown in fig. 5.A-F. As might be expected, a more prominent increase in degradation is demonstrated when these preparations are stored in solution at 4 °C.

The values estimated for impurities, including the deamidated forms of somatropin may be useful in considerations for setting limits in a pharmacopoeia monograph on somatropin injection. The following estimates for the content of deamidated forms (I_4) after storage were obtained for the batches of somatropin preparations in solution marketed in Norway (number of months until expiry date indicated in parenthesis): 9.6 % (0), 10.0 % (2), 3.5 % (3), 6.7 % (8) and 6.2 % (7), see table 3.B. Regarding degradation it has been reported that also the deamidated forms of somatropin have growth promoting activity [1, 15, 16]. Regarding the relatively high content of degraded forms demonstrated in the somatropin preparations, assurance must be established that their activity is qualitatively and quantitatively similar to the activity of the principal form of somatropin, and that no additional unwanted effects are developed. On the other hand it is not acceptable that the active substance of a medicinal product shifts from a pure substance to an inhomogeneous mixture of related chemical entities during its shelf life.

Additional degradation products and also changes in their relative content during storage, have been considered during these stability studies (tables 3.A and B). However, due to batch variations in Norditropin SimpleXx is a complicating factor, and further assessment is needed.

Table 3.A Content of charged somatropin variants of initial test solutions (%).

Product	Expiry	Date	I_1	I_2	I_3	I_4	I_5	I_{Total}
Norditropin SimpleXx:	09/2008	21.11.07	1.67	0.57	0.61	6.55	0.00	9.40
	11/2008	05.12.07	1.29	0.22	0.52	6.91	0.00	8.94
	12/2008	28.02.08	1.20	0.35	0.16	1.24	0.07	3.02
	05/2009	03.03.08	1.23	0.24	0.30	4.27	0.00	6.04
NutropinAq:	04/2009	19.12.07	2.92	0.00	0.39	3.06	0.00	6.37
Genotropin:	09/2008	03.12.07	0.33	0.00	0.06	0.29	0.00	0.68
	09/2009	04.12.07	0.28	0.00	0.08	0.22	0.00	0.58
Humatrope :	01/2009	26.11.07	0.30	0.00	0.45	1.80	0.00	2.55
Saizen:	05/2008	27.11.07	0.08	0.04	0.46	1.10	0.51	2.19
	10/2009	30.11.07	0.25	0.08	0.17	1.00	0.58	2.08
Zomacton:	11/2008	24.12.07	0.35	0.48	0.44	2.02	0.00	3.29

I_1, I_2 (cleaved form), I_3 (Gln-18 somatropin), I_4 (deamidated forms), I_5 (deamidated form).

Table 3.B Content of charged somatropin variants after storage at 4 ^{o}C (%).

Product	Expiry	No. of days	I_1	I_2	I_3	I_4	I_5	I_{Total}
Norditropin SimpleXx:	09/2008	275	0.79	0.34	0.41	9.63	0.03	11.20
	11/2008	273	0.80	0.14	0.40	9.95	0.00	11.29
	12/2008	187	1.12	0.27	0.21	3.50	0.04	5.14
	05/2009	188	1.00	0.26	0.32	6.68	0.00	8.26
NutropinAq:	04/2009	260	1.39	0.06	0.54	6.22	0.00	8.21
Genotropin:	09/2008	285	0.10	0.16	0.43	9.26	0.00	9.95
	09/2009	285	0.18	0.00	0.39	8.38	0.00	8.95
Humatrope:	01/2009	284	0.00	0.00	0.90	15.46	0.00	16.36
Saizen:	05/2008	283	0.00	0.93	2.04	19.98	0.00	22.95
	10/2009	281	0.00	0.14	1.85	17.26	0.04	19.29
Zomacton:	11/2008	257	0.00	0.64	0.76	11.55	0.00	12.95

Fig. 6 shows the eletropherograms for the initial test solutions of two strengths of a somatropin preparation in solution (Norditropin SimpleXx), and two strengths of one of a freeze-dried preparation (Saizen) before storage, demonstrating the distribution of the charged variants of somatropin. Concerning the latter preparation, an additional peak eluting just after the principal peak, in front of Gln-18 somatropin (I_3), was observed. This component, not described in the pharmacopoeias, designated $I_?$, may be due to the different production method for this somatropin preparation.

Fig.4 Degradation of somatropin preparations in solution after dilution/preparation of test solution 1.0 mg/ml:
(A) Norditropin SimpleXx 10mg/1.5ml batch TU60933 exp.09/2008, (B) Norditropin SimpleXx 5mg/1.5ml batch TU60855 exp.11/2008, (C) Norditropin SimpleXx 5mg/1.5ml batch TR40418 exp.13dec 2008, (D) Norditropin SimpleXx 5mg/1.5ml batch TU61386 exp.05/2009, (E) NutropinAq 10mg/2ml batch SOO5(N67268) exp.04/2009.

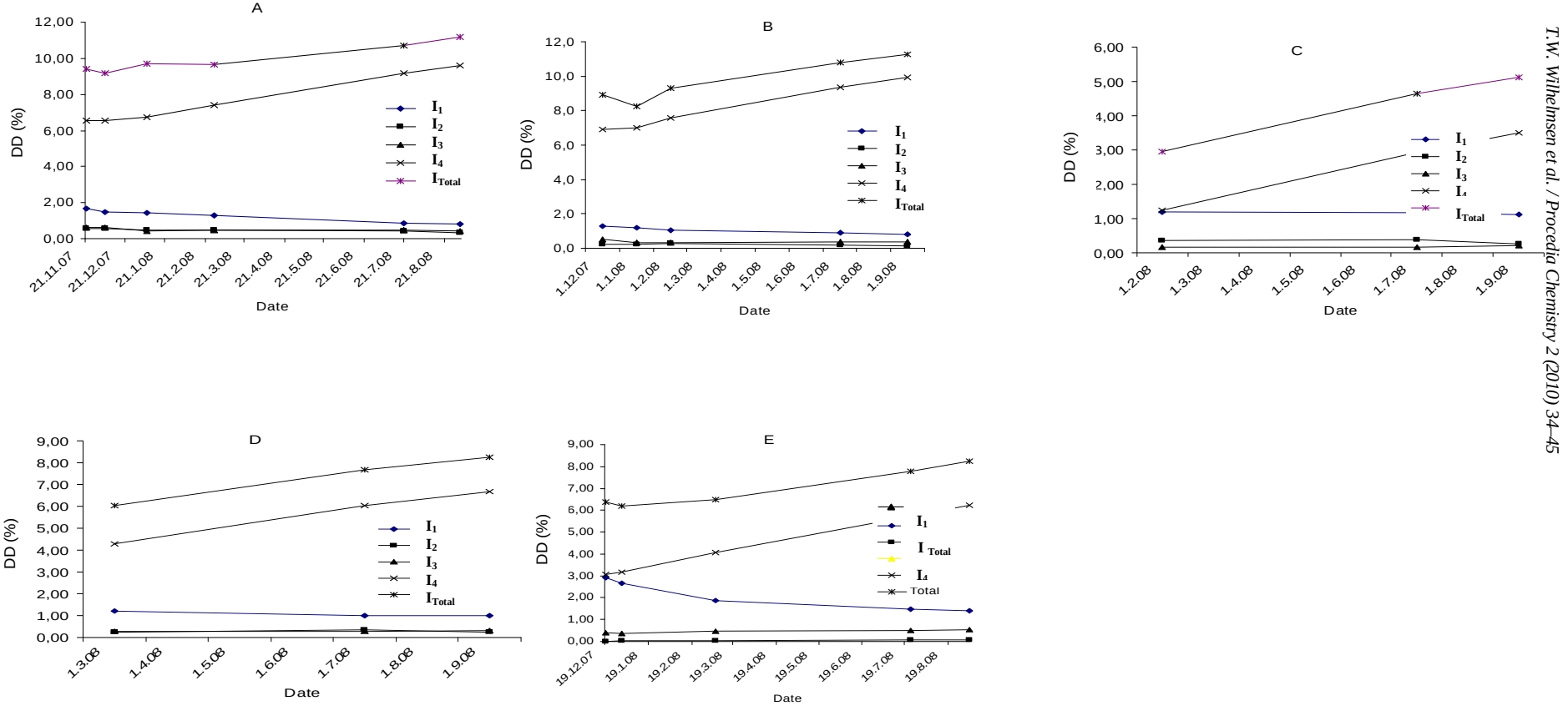


Fig.5 Degradation of freeze-dried somatropin preparations after reconstitution and preparation of test solution 1.0 mg/ml:
(A) Genotropin 0.4mg batch PO2483 exp.09/2008, (B) Genotropin 5mg batch PO1548 exp.09/2009, (C) Humatrope 12mg batch A324817 exp.01/2009, (D) Saizen 1.33mg batch YO3A8242 exp.05/2008, (E) Saizen 8mg batch YO5B4666 exp.10/2009, (F) Zomacton 4mg batch CA0760G exp.11/2008.

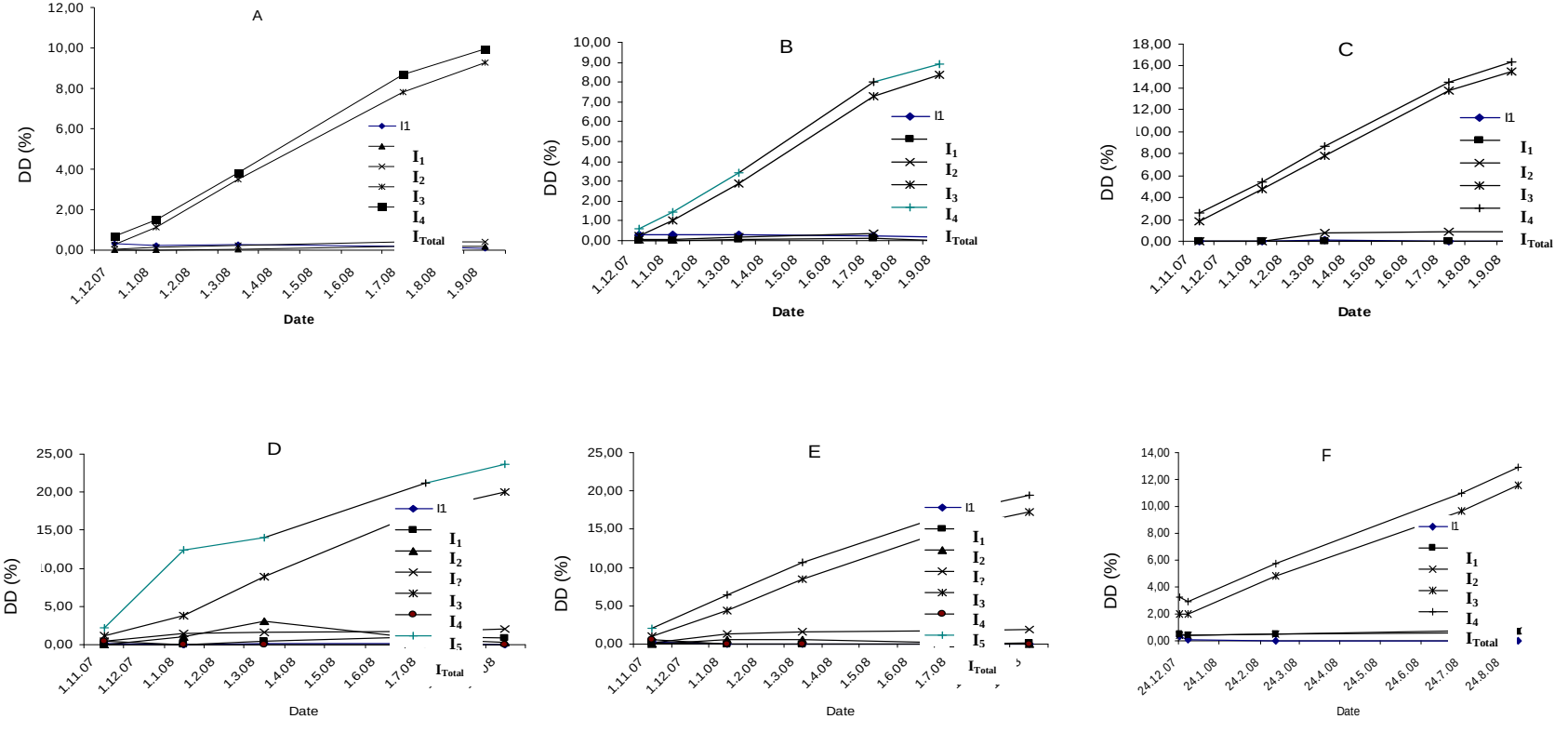
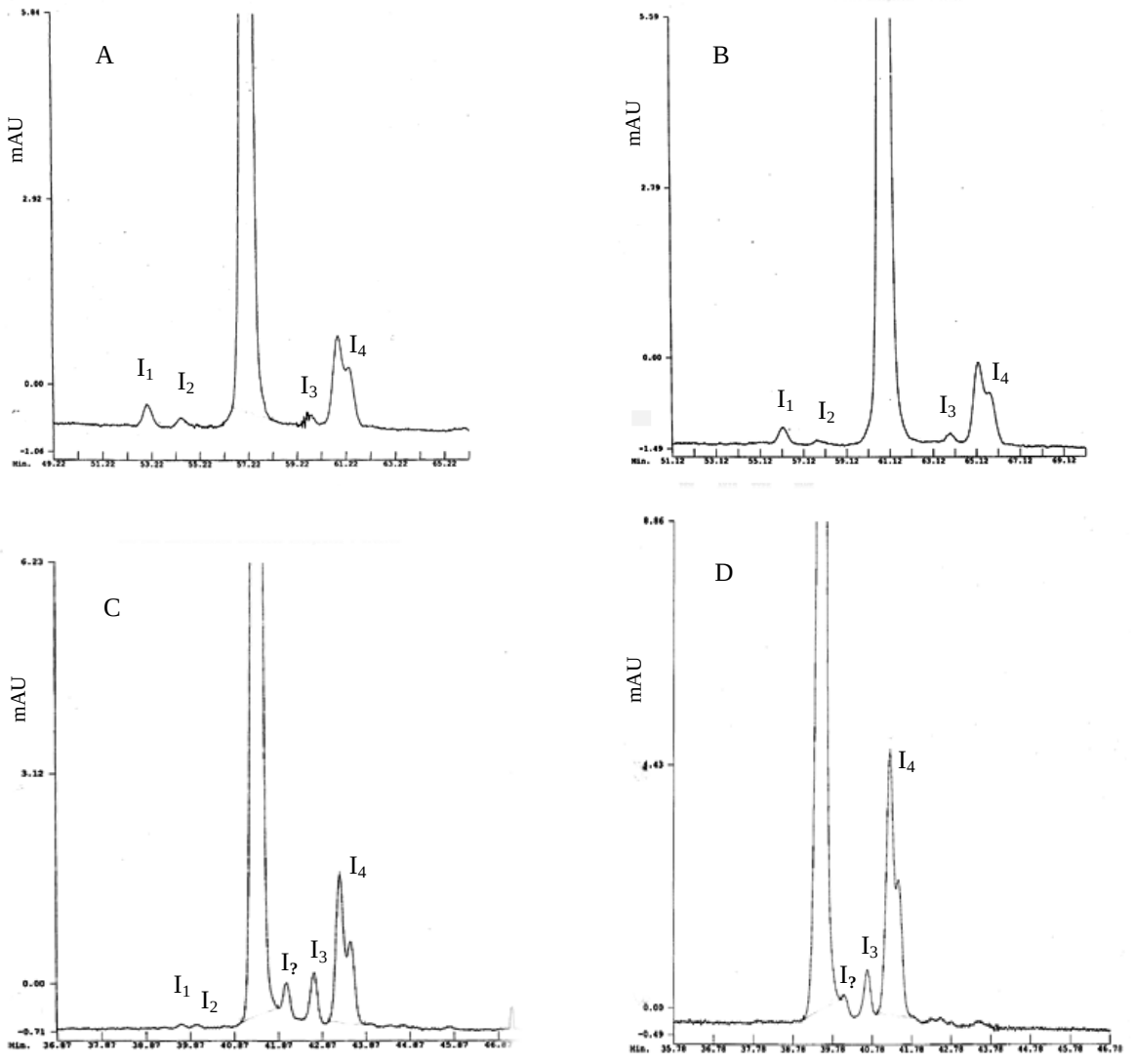


Fig.6 Electropherogram of initial test solutions, 1.0 mg/ml.
(A) Norditropin SimpleXx 10mg/1.5ml, exp.09/2008.
(B) Norditropin SimpleXx 5mg/1.5ml, exp.12/2008.
(C) Saizen 1.33 mg, exp. 05/2008
(D) Saizen 8 mg, exp.11/2008.



I = impurity.
I_1, I_2 (cleaved form), $I_?$ (unknown), I_3 (Gln-18 somatropin), I_4 (deamidated forms).

The rhGH profile shown in fig. 6.A and B is produced in E. coli, however, somatropin in Saizen (fig. 6.C and D) is made in a mammalian cell line, and as a consequence, post-translational modifications other than the ones occurring in bacteria are likely to happen. For instance, mammalian cells are able to glycosylate recombinant proteins while proteins produced in E. coli do not contain sugar.

The additional peak seen in fig. 6.C and D in the rhGH profile from mammalian cells compared to the profiles of somatropin produced in E. coli, might represent a glycosylated form [1, 17]. The existence of a glycosylated variant from the human pituitary gland has previously been shown, probably containing O-linked sugar [17], and glycosylated forms of both chicken and murine [18, 19] GH have been reported.

4. Conclusion

The results demonstrate the existence of many rhGH variants in the somatropin preparations on the market. Peaks corresponding to both deamidated, cleaved and also unknown variants are present in the profiles after CZE. In addition an increasing content of modified/degraded forms have been shown in the recombinant somatropin solutions after storage at 4 ^{o}C.

A pharmacopoeia standard also for somatropin presented as an injection solution, including acceptance limits for the concerned impurities, is needed.

References

1. G.P. Baumann., Growth Hormone & IGF Research 19 (2009) 333.
2. C.H. Li and H. Papkoff, Sience 124 (1956) 1293.
3. H.D. Niall, Nat. New Biol. 230 (1971) 90.
4. U.J. Lewis, R.N. Singh, L.F. Bonewald, L.J. Lewis, W.P. Vanderlaan., Endocrinology 104 (1979) 1256.
5. S. Hjertén, Chromatogr. Rev. (1997) 122.
6. V. Dolnik. Capillary electrophoresis of proteins 2005-2007. Electrophoresis 29 (2008):143-156.
7. V. Kasicka, Electrophoresis 29 (2008) 179.
8. J.R. Catai, J.S. Torano, P.M.J.M. Jongen, G.J. de Jong, G.W. Somsen, J. Chromatogr. B 852 (2007) 160.
9. T.K. Jørgensen, L.H. Bagger, J. Christensen, G.H. Johnsen, J.R. Faarbaek, L. Jørgensen, B.S. Welinder, J. Chromatogr. A 817 (19998) 205.
10. T.M. Mcnerney, S.K. Watson, J.H. Sim, R.L. Bridenbaugh, J. Chromatogr. A 744 (1996) 223.
11. Capillary electrophoresis, European Pharmacopoeia (2.2.47).
12. Erythropoietin concentrated solution, European Pharmacopoeia (01/2008:1316).
13. Somatropin for injection, European Pharmacopoeia (01/2008:0952).
14. Somatropin for injection, British Pharmacopoeia (2007, vol. III).
15. G. Bauman, Endocr. 12 (1991) 424.
16. U.J. Lewis, Trends Endocrinol. Metab. 3 (1992) 117.
17. L.S. Haro, U.J. Lewis, M. Garcia, J. Bustamante, A.O. Martinez, N.C. Ling, Biochemical and Biophysical Research Communication 228 (1996) 549.
18. L.R. Berghman, P. Leene, E. Decuipere, E.R. Kuhn, F. Vandesande, Gen. Comp. Endocrinol, 68 (1987) 408.
19. Y.N. Sinha and B.P. Jacobsen, Biochem. Biophys. Res. Commun. 145 (1987) 1368.